

# Molecular genetic linkage maps for allotetraploid *Leymus* wildryes (Gramineae: Triticeae)

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**Abstract:** Molecular genetic maps were constructed for two full-sib populations, TTC1 and TTC2, derived from two *Leymus triticoides* × *Leymus cinereus* hybrids and one common *Leymus triticoides* tester. Informative DNA markers were detected using 21 *EcoRI*-*MseI* and 17 *PstI*-*MseI* AFLP primer combinations, 36 anchored SSR or STS primer pairs, and 9 anchored RFLP probes. The 164-sib TTC1 map includes 1069 AFLP markers and 38 anchor loci in 14 linkage groups spanning 2001 cM. The 170-sib TTC2 map contains 1002 AFLP markers and 36 anchor loci in 14 linkage groups spanning 2066 cM. Some 488 homologous AFLP loci and 24 anchor markers detected in both populations showed similar map order. Thus, 1583 AFLP markers and 50 anchor loci were mapped into 14 linkage groups, which evidently correspond to the 14 chromosomes of allotetraploid *Leymus* ( $2n = 4x = 28$ ). Synteny of two or more anchor markers from each of the seven homoeologous wheat and barley chromosomes was detected for 12 of the 14 *Leymus* linkage groups. Moreover, two distinct sets of genome-specific STS markers were identified in these allotetraploid *Leymus* species. These *Leymus* genetic maps and populations will provide a useful system to evaluate the inheritance of functionally important traits of two divergent perennial grass species.

**Key words:** AFLP, perennial grasses, RFLP, STS, SSR.

**Résumé :** Des cartes génétiques ont été produites pour deux populations soeurs, TTC1 et TTC2, chacune issue du croisement entre un hybride *Leymus triticoides* × *Leymus cinereus* et une même lignée test du *Leymus triticoides*. Des marqueurs polymorphes ont été obtenus en examinant 21 combinaisons d'amorces AFLP *EcoRI*-*MseI*, 17 combinaisons d'amorces AFLP *PstI*-*MseI*, 36 paires d'amorces spécifiques d'un locus (microsatellites ou STS) et 9 sondes RFLP. La carte TTC1 ( $N = 164$ ) comprend 1069 marqueurs AFLP et 38 locus d'ancrage, lesquels forment 14 groupes de liaison totalisant 2001 cM. La carte TTC2 ( $N = 170$ ) compte 1002 marqueurs AFLP et 24 marqueurs d'ancrage, le tout formant 14 groupes de liaison et totalisant 2066 cM. Quelque 488 locus AFLP homologues et 24 marqueurs d'ancrage étaient communs aux deux cartes et ils étaient placés dans un ordre semblable. Au total, 1583 marqueurs AFLP et 50 marqueurs d'ancrage formaient 14 groupes de liaison correspondant évidemment aux 14 chromosomes chez le *Leymus* allotétraploïde ( $2n = 2x = 28$ ). De la syténie impliquant au moins deux marqueurs d'ancrage, provenant de chacun des sept chromosomes homéologues de blé et de l'orge, a été observée chez 12 des 14 groupes de liaison du *Leymus*. De plus, deux marqueurs STS spécifiques d'un génome ont été identifiés chez ces *Leymus* allotétraploïdes. Ces cartes génétiques et ces populations seront utiles pour évaluer l'hérédité de caractères importants chez deux espèces de graminées pérennes assez différentes.

**Mots clés :** AFLP, graminées pérennes, RFLP, STS, SSR.

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**Abbreviations:** AFLP, amplified fragment length polymorphism; cM, centiMorgan; RFLP, restriction fragment length polymorphism; SSR, simple-sequence repeat; STS, sequence-tagged site.

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## Introduction

The genus *Leymus* comprises about 30 long-lived perennial grass species distributed across inland and coastal regions of Asia, Europe, and North and South America. Several *Leymus* species are cultivated as forage and (or) conservation grasses in Eurasia and North America (Asay and Jensen 1996). *Leymus* is also one of several perennial Triticeae genera used in wide-hybridization wheat breeding (Jiang et al. 1994). Alien gene transfer of salinity tolerance and disease resistance from *Leymus* to wheat has been the main objective of these efforts. Conversely, other wide-hybridization breeding efforts involving *Leymus* and wheat aim to improve *L. arenarius* as a perennial grain crop in high-latitude regions unsuitable for conventional cereal crops (Anamthawat-Jónsson et al. 1997). All things considered, *Leymus* includes a wide diversity of naturally important grasses that have potential value in agriculture.

Perennial grasses display divergent adaptations in terms of growth habit, phenology, salinity tolerance, seed dormancy, cold-temperature growth, and many other traits. However, relatively little is known about the genetic control of functional traits in perennial grasses per se. High-density genetic maps are becoming increasingly useful in fundamental and applied research aiming to (i) examine and compare the genetic basis of complex traits in divergent plant species, (ii) locate and identify functionally important genes, and (iii) facilitate marker-assisted selection for plant improvement and other plant genetic research (Paterson 1997; Vuylsteke et al. 1999). Although genomic research in barley and wheat (Lagudah et al. 2001) may facilitate genetic research in the perennial Triticeae, many of the long-lived grasses are unwieldy polyploids with complex modes of reproduction and inheritance.

The perennial Triticeae have been the subject of considerable cytogenetic research, stemming in part from wide-hybridization breeding efforts involving wheat. More than half of all *Leymus* species are allotetraploids ( $2n = 4x = 28$ ). Octoploid ( $2n = 8x = 56$ ) and duodecaploid ( $2n = 12x = 84$ ) variants may arise from interspecific hybrids (Anamthawat-Jónsson and Bödvarsdóttir 2001) or autoduplication within species. Based on chromosome pairing in triploid hybrids, possible diploid ancestors of allotetraploid *Leymus* were initially identified as *Psathyrostachys* and *Thinopyrum* (Dewey 1970; Dewey 1984; Löve 1984). While at least one *Psathyrostachys* genome (*Ns*) has been substantiated in *Leymus* (Zhang and Dvorak 1991; Wang and Jensen 1994; Wang et al. 1994; Anamthawat-Jónsson and Bödvarsdóttir 2001), cytogenetic and molecular data have refuted this putative genome relationship between *Leymus* and *Thinopyrum* (Zhang and Dvorak 1991; Wang and Jensen 1994). The consensus of several researchers has been to reserve designation of one *Leymus* genome, *Xm*, as unknown (Wang and Jensen 1994; Wang et al. 1994; Sun et al. 1995). However, hybridization of *Leymus* DNA with repeated nucleotide sequences from other Triticeae species suggested that *Leymus* is a segmental autoploid, *Ns<sub>1</sub>Ns<sub>1</sub>Ns<sub>2</sub>Ns<sub>2</sub>*, derived from two distinct *Psathyrostachys* species (Zhang and Dvorak 1991). Similarly, comparisons of 18S–26S ribosomal DNA sequences between *Leymus* and *Psathyrostachys* are more similar than

comparisons of 18S–26S ribosomal DNA sequences among different species within these two genera (Anamthawat-Jónsson and Bödvarsdóttir 2001). Although *Leymus* and *Psathyrostachys* are circumscribed by species with homologous chromosomes (Dewey 1984; Löve 1984), genome distinctions within these genera may not be fully appreciated (Anamthawat-Jónsson and Bödvarsdóttir 2001). The nuclear DNA content of the allotetraploid *Leymus* species range from 19.4 to 22.8 pg/2C, which is approximately midway between diploid barley (10.7 pg/2C) and hexaploid wheat (34.7 pg/2C) (Vogel et al. 1999). However, the nuclear DNA content of *Psathyrostachys* species ranges from 15.5 to 17.9 pg/2C (Vogel et al. 1999). Therefore, the *Leymus* genome is physically smaller than expected based on the combination of any two *Psathyrostachys* species examined by Vogel et al. (1999). However, the DNA content of alloctetraploid western wheatgrass (*Pascopyrum smithii*) is also smaller than expected based on its putative allotetraploid ancestors thickspike wheatgrass (*Elymus lanceolatus*) and creeping wildrye (*Leymus triticoides*) (Vogel et al. 1999). In any case, allotetraploid *Leymus* species with disomic inheritance may provide a novel and suitable system to expand comparative genomic research in the Triticeae.

Allotetraploid *L. triticoides* and *L. cinereus* are closely related, but morphologically distinct North American range grasses. *Leymus triticoides* is strongly rhizomatous and adapted to poorly drained alkaline sites, primarily within the western United States. *Leymus cinereus* is a tall bunch grass widely distributed from British Columbia to Saskatchewan in the north and California to Arizona and Colorado in the south. Most populations of *L. cinereus* and *L. triticoides* are allotetraploids, however octoploid forms of *L. cinereus* are typical in the Pacific northwest. Although both species show the relatively high salinity tolerance characteristic of this genus, *L. triticoides* seedlings and tillers display better survival when grown in silica sand and sustained by nutrient solutions with increasing salt concentrations (results unpublished). Likewise, unusually high levels of seed dormancy are characteristic of *L. triticoides* (Knapp and Wiesner 1978). Thus, highly divergent forms of natural seed dormancy are also maintained between *L. triticoides* and *L. cinereus*. Interestingly, recalcitrant seed dormancy seems to coincide with relatively dark anthocyanin coloration in the seeds of *L. triticoides* and closely related western wheatgrass (*P. smithii*). Red seed color and coat-imposed seed dormancy are simply inherited traits in wheat, evidently controlled by the same gene located on homoeologous chromosome 3 (Flintham and Humphray 1993; Nelson et al. 1995; Flintham and Gale 1996). In any case, artificial hybrids of *L. cinereus*, *L. triticoides*, and other North American *Leymus* species display regular meiosis and stainable pollen (Stebbins and Walters 1949; Dewey 1972; Hole et al. 1999). Although some *L. cinereus* × *L. triticoides* hybrids fail to produce seed (Stebbins and Walters 1949; Dewey 1972; Hole et al. 1999), authors of this study have observed highly variable levels of pollen and (or) seed fertility among other artificial hybrids of *L. cinereus* and *L. triticoides*. Both *L. cinereus* and *L. triticoides* are highly self sterile (Jensen et al. 1990) and hybridize in nature. Thus, *L. cinereus* and *L. triticoides* may provide a particularly useful genetic system to evaluate the

inheritance of functional traits such as growth habit, phenology, salinity tolerance, and seed dormancy in perennial grasses.

The AFLP technique (Vos et al. 1995) is a robust and efficient method of constructing high-density molecular genetic maps in higher plant species with complex genomes (Vuylsteke et al. 1999). The objectives of this research were to (*i*) construct high-density AFLP maps and compare the inheritance of AFLP markers from two interspecific hybrids of allotetraploid *L. triticoides* × *L. cinereus*, (*ii*) test the identity of these putative AFLP linkage groups using RFLP, SSR, and STS markers that have been genetically mapped to homoeologous chromosomes of barley, wheat, and other cereal crop species, and (*iii*) investigate the phylogenetic origin and identity of the allopolyploid *Leymus* genome by comparing homoeologous nuclear DNA sequences of *Leymus*, *Psathyrostachys*, and other *Triticeae* species. We anticipate that these genetic maps and segregating populations will be a useful starting point to investigate divergent adaptations of *L. cinereus*, *L. triticoides*, and other perennial *Triticeae*.

## Materials and methods

### Plant material and DNA isolation

Full-sib mapping populations TTC1 and TTC2 were derived from one *L. triticoides* accession 641 plant (T tester) pollinated by two different *L. triticoides* accession 641 × *L. cinereus* accession 636 F<sub>1</sub> hybrids (TC1 and TC2). Because *L. cinereus* and *L. triticoides* are outcrossing, accessions 636 and 641 and the derived hybrids are genetically heterogeneous. Thus, TC1 and TC2 hybrids are genetically different. Approximately 50 T-tester spikes were ensconced in pollination bags before anthesis (3–5 spikes per closure) and labeled according to the TC1 or TC2 pollen source. Virtually all seeds were germinated and 178 plants were randomly selected from each of the TTC1 (T tester × TC1) and TTC2 (T tester × TC2) seedling populations.

Accession 636 was received from the Agriculture Research Centre, Lethbridge, Alta., and presumably originates from a natural population in Alberta or Saskatchewan. Accession 641 was collected by Dr. Kay H. Asay from a natural population near Jamieson, Oreg. The F<sub>1</sub> hybrids were derived from controlled crosses between heterogeneous populations of accession 636 and accession 641; however, the exact parental genotypes of the TC1 and TC2 hybrids were not preserved. Source clones of the T tester, TC1, TC2, 178 TTC1, and 178 TTC2 genotypes are currently maintained at the United States Department of Agriculture – Agriculture Research Service Forage and Range Research Laboratory (Logan, Utah) and two clonally replicated blocks at the Agriculture Experiment Station field site in Richmond, Utah. Plant genomic DNA was obtained from 384 leaf samples including 8 accession 636 plants, 8 accession 641 plants, 4 T-tester clones, 4 TC1 clones, 5 TC2 clones, 178 TTC1 siblings, and 178 TTC2 siblings using the DNeasy® plant DNA isolation kits (Qiagen Inc., Valencia, Calif.).

### AFLP procedures

AFLP reactions were carried out as described by Vos et al. (1995) and Vuylsteke et al. (1999) using two different restriction enzyme combinations, *Eco*RI–*Mse*I and *Pst*I–*Mse*I.

The adapter sequences specific for those enzymes were prepared by standard synthesis of the following oligonucleotides:

*Eco*RI adapter: forward, 5'-CTCGTAGACTGCGTACC-3'; reverse, 3'-CATCTGACGCATGGTAA-5';

*Pst*I adapter: forward, 5'-CTCGTAGACTGCGTACAT-GCA-3'; reverse, 3'-CATCTGACGCATGT-5';

*Mse*I adapter: forward, 5'-GACGATGAGTCCTGAG-3'; reverse, 3'-GTAGTCACGTACGC-5'.

The first PCR amplification of restriction fragments with one selective nucleotide was performed using similar preparations of the following oligonucleotides:

*Eco*RI (+1), 5'-GACTGCGTACCAATTCA-3';

*Pst*I (+1), 5'-GACTGCGTACATGCAGA-3';

*Mse*I (+1), 5'-GATGAGTCCTGAGTAAC-3'.

The second PCR amplification of restriction fragments was performed using the following combinations:

*Eco*RI (+3), 5'-GACTGCGTACCAATTCA-3';

*Pst*I (+3), 5'-GACTGCGTACATGCAGA-3';

*Mse*I (+3), 5'-GATGAGTCCTGAGTAAC-3';

with three selective nucleotides as described in Table 1. The 5' nucleotide of the *Eco*RI (+3) and *Pst*I (+3) selective amplification primers was labeled with 6-carboxyfluorescein (6-FAM).

Fluorescent-labeled DNA fragments were size fractionated using an ABI Prism 3100 genetic analyzer with 50-cm capillaries, POP-6 polymer, and rhodamine X (ROX)-labeled GS400HD internal size standards according to the manufacturer instructions (PE Applied Biosystems Inc., Foster City, Calif.). The segregation of fluorescent AFLP, SSR, and STS PCR markers was analyzed using a combination of Genescan software (PE Applied Biosystems) and Genographer version 1.5 (Benham et al. 1999) software.

### STS and SSR protocols

PCRs were carried out in a total volume of 50 µL. Each reaction contained 1.0 U *Taq* polymerase, 6 pmol each primer, 200 µM dNTPs, 3.0 mM MgCl<sub>2</sub>, 10 pmol fluorescent-labeled [R110]dCTP, 1× PCR buffer, and between 50 and 100 ng of total genomic DNA. Cycling parameters consisted of 5 min at 94°C, followed by 38 cycles at 94°C for 1 min, 50–60°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 5 min.

A large number of STS and SSR primers were designed from previously published wheat and barley sequences and initially tested on the TC1, TC2, and T-tester parental DNA samples. The PCR amplicons were fractionated by 3% w/v agarose gel electrophoresis with 100- to 1000-bp size standards. Highly specific amplicons within the expected size range were also fractionated and analyzed by capillary electrophoresis, as described for AFLP markers above, with GS400HD(ROX) or GS500(ROX) internal size standards. Before electrophoresis, the STS amplicons were digested with a panel of 10–20 different restriction enzymes, each having unique 4- or 5-bp recognition sequences. Thus, single-nucleotide polymorphisms (SNPs) or insertion-deletion (indel) variants were detected as RFLPs of the STS amplicons.

**Table 1.** Summary of amplified fragment length polymorphisms (AFLPs) genetically mapped among 14 linkage groups in *Leymus* mapping populations TTC1 and (or) TTC2, amplified by 21 *EcoRI*-*MseI* and 17 *PstI*-*MseI* primer combinations with three selective nucleotides.

Primer combination(s)	Selective nucleotides	TTC1	TTC2	TTC1 and TTC2 (common)	TTC1 and TTC2 (corrected total)
E36M48	E(ACC)/M(CAC)	25	28	11	42
E36M49	E(ACC)/M(CAG)	28	25	9	44
E36M50	E(ACC)/M(CAT)	14	15	7	22
E36M59	E(ACC)/M(CTA)	29	22	9	42
E36M61	E(ACC)/M(CTG)	45	41	23	63
E36M62	E(ACC)/M(CTT)	35	34	18	51
E37M47	E(ACG)/M(CAA)	34	32	16	50
E37M49	E(ACG)/M(CAG)	24	30	10	44
E37M60	E(ACG)/M(CTC)	34	31	15	50
E37M62	E(ACG)/M(CTT)	29	28	14	43
E38M47	E(ACT)/M(CAA)	32	30	17	45
E38M49	E(ACT)/M(CAG)	35	22	15	42
E38M59	E(ACT)/M(CTA)	25	29	13	41
E38M60	E(ACT)/M(CTC)	35	34	19	50
E41M47	E(AGG)/M(CAA)	35	36	17	54
E41M48	E(AGG)/M(CAC)	32	40	15	57
E41M49	E(AGG)/M(CAG)	30	27	15	42
E41M59	E(AGG)/M(CTA)	33	33	9	57
E41M60	E(AGG)/M(CTC)	35	40	15	60
E41M61	E(AGG)/M(CTG)	41	49	23	67
E41M62	E(AGG)/M(CTT)	27	35	13	49
<b>EM total</b>		657	661	303	1015
P33M47	P(AAG)/M(CAA)	28	19	11	36
P33M48	P(AGG)/M(CAC)	30	24	14	40
P33M50	P(AGG)/M(CAT)	26	25	11	40
P33M59	P(AGG)/M(CTA)	20	15	9	26
P33M60	P(AGG)/M(CTC)	23	23	14	32
P33M61	P(AGG)/M(CTG)	24	19	12	31
P33M62	P(AGG)/M(CTT)	31	26	13	44
P35M49	P(ACA)/M(CAG)	20	14	7	27
P35M50	P(ACA)/M(CAT)	18	18	10	26
P35M59	P(ACA)/M(CTA)	16	12	6	22
P35M60	P(ACA)/M(CTC)	20	16	9	27
P35M61	P(ACA)/M(CTG)	22	14	9	27
P35M62	P(ACA)/M(CTT)	34	31	19	46
P42M61	P(AGT)/M(CTG)	24	17	11	30
P42M62	P(AGT)/M(CTT)	16	16	3	29
P44M49	P(ATC)/M(CAG)	23	25	10	38
P44M62	P(ATC)/M(CTT)	37	27	17	47
<b>PM total</b>		412	341	185	568
<b>EM+PM total</b>		1069	1002	488	1583

### RFLP markers

The CDO460, CDO1401, BCD250, BCD421, BCD1130, BCD1150, BCD1532, BCD1562, and BCD1707 clones (Heun et al. 1991) were obtained from the GrainGenes Probe Repository at the USDA-ARS Western Regional Research Center (Albany, Calif.). Corresponding RFLP loci in *Leymus* TTC1 and TTC2 populations were detected using *EcoRI* as described in *Current protocols in molecular biology* (Ausubel 1978) with minor modifications by Biogenetic Services, Inc. (unpublished).

### Genetic analyses and map construction

Genetic linkage maps were constructed based on the segregation of DNA markers among gametes from the TC1 and TC2 hybrids using the doubled-haploid (DH) population model of JoinMap 3.0 (van Ooijen and Voorrips 2001). In practice, this analysis was based on DNA fragments present in the TC1 and (or) TC2 hybrids, absent in the T-tester, and segregating in the TTC1 and (or) TTC2 populations. These markers have a 1:1 expected segregation ratio (assuming disomic inheritance of monoallelic fragments) corresponding

to gamete genotypes. A two-phase approach was used to construct these high-density linkage maps (Vision et al. 2000). The first phase was construction of a high-confidence and high-density framework of AFLP markers. All AFLPs with more than 10% missing data were dropped without any further analysis. Linkage groups were evaluated by increments of 2 LOD values from 4 to 40. Linkage analyses were performed using (i) unknown linkage phase, and (ii) a mixture of inferred and unknown linkage phase designations. Although the exact parental genotypes of the TC1 and TC2 hybrids were not available, the linkage phase of most markers could be inferred by comparison of the eight accession 636 genotypes and the eight accession 641 genotypes. The relative order, goodness of fit, and distances among AFLP markers was determined by three cycles of map construction. The marker maps were rippled with the sequential addition of each marker. Map distance among markers was determined using Kosambi's mapping function. The first two cycles placed well-fitting markers with  $\chi^2$  values less than 5.00. A third cycle forced remaining markers onto the map.

The second phase of this mapping effort was to add RFLP, STS, and SSR anchor markers by segregation analysis of 96 genotypes from one or both populations. These 96 genotypes were selected on the basis of recombination detected in the AFLP map. These markers were subsequently integrated into the AFLP map as the best possible fit. Maps were drawn using MapChart 2.0 (Voorrips 2001). The *L. cinereus* and *L. triticoides* germplasm contributions in TTC1 and TTC2, number of singletons, and deduced number of recombinations per gamete was evaluated using GGT: Graphical GenoTyping (van Berloo 1999). Amino acid and DNA sequences were aligned using Clustal W (Thompson et al. 1994). Parsimony analysis of the DNA sequences was performed using the branch-and-bound search method of PAUP\* (Swofford 1998).

## Results and discussion

### AFLP analysis and framework map

Thirty-eight AFLP primer combinations (Table 1) amplified approximately 1390 fragments from the TC1 hybrid and 1410 fragments in the TC2 hybrid, which were absent in the T-tester and segregating in each of the corresponding TTC1 and TTC2 populations. Approximately 76% of these scored markers were fixed or segregating among a sample of eight accession 636 genotypes, but absent among the sample of eight accession 641 genotypes. Conversely, about 10% of the scored markers were absent among the sample of accession 636 genotypes, but segregating among the accession 646 genotypes. The remaining 14% were fixed or segregating among the accession 636 samples and segregating among the accession 641 samples. Thus, linkage phase was readily inferred for approximately 86% of the scored markers based on the pedigree genotypes. The linkage-phase configuration of all markers computed by JoinMap was virtually identical with or without our inferences based on pedigree genotypes. Overall, nearly 90% of the scored AFLP markers were evidently coupled in accession 636 as computed by Joinmap. Thus, most of the mapped DNA fragments (below) originate

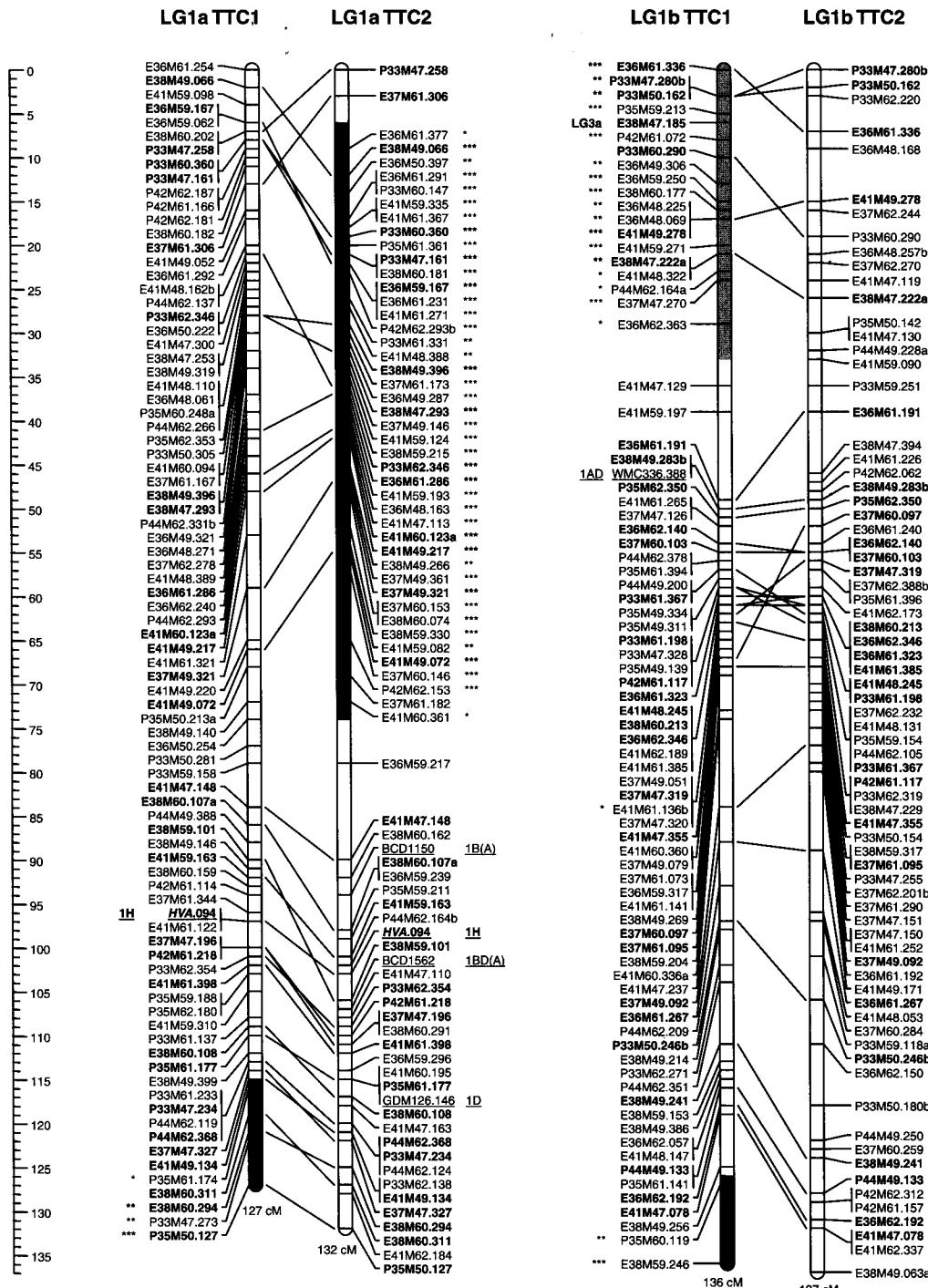
from *L. cinereus*. A relatively small number of other markers were absent in each of the TC1 and TC2 hybrids, present in the T tester, and segregating approximately 1:1 in the corresponding TTC1 and TTC2 populations. In sufficient quantity, these markers could be used to construct a third map based on the *L. triticoides* T tester. Rarely, DNA fragments were present in each of the TC1 and TC2 hybrids, present in the T tester, and segregating approximately 3:1 in the corresponding TTC1 and TTC2 populations. Albeit difficult, these markers could in theory be used to integrate the interspecific TC1 and TC2 maps with the T-tester map (Maliepaard et al. 1997). However, an insufficient number of markers were available to construct and integrate the *L. triticoides* T-tester map and we did not score any of the segregating DNA fragments that were present in the T tester. The primary objective of these efforts was to examine interspecific differences between *L. cinereus* and *L. triticoides* by testcross analysis of the TC1 and TC2 gametes.

Six progeny genotypes of each population were virtually identical to the T tester, albeit missing some of the T-tester DNA fragments that segregated in TTC1 and (or) TTC2. These 12 genotypes undoubtedly resulted from self-fertilization and were omitted from any further analysis. Moreover, eight TTC1 and two TTC2 genotypes with more than 10% missing data were also excluded from the linkage analysis. Thus, mapping populations TTC1 and TTC2 comprise 164 and 170 segregating genotypes, respectively.

A total of 1390 and 1410 markers were scored in the TTC1 and TTC2 populations, respectively. A majority of the AFLP markers (Table 1) were readily partitioned into 14 well-defined linkage groups (Fig. 1) that evidently correspond to the 14 chromosomes of allotetraploid *Leymus*. Critical values required to separate linkage groups in population TTC1 were as follows: LOD = 2: LG3a, LG4Xm, and LG5Xm; LOD = 4: LG1b, LG2b, and LG7b; LOD = 6: LG1a, LG2a, LG3b, LG4Ns, LG5Ns, and LG7a; LOD = 8: LG6a and LG6b. Likewise, minimum thresholds for distinguishing linkage groups in population TTC2 were as follows: LOD = 2: LG7a and LG7b; LOD = 4: LG2a and LG4Xm; LOD = 6: LG1a, LG3a, LG5Ns, and LG5Xm; LOD = 8: LG2b, LG3b, LG4Ns; LOD = 10: LG1b, LG6a, and LG6b. However, approximately 250 markers separated out individually or in small groups, from each population, at lower LOD thresholds. These markers, which displayed ambiguous associations with two or more linkage groups, were excluded from further analysis. Interestingly, the LG6a and LG6b groups were somewhat difficult to separate in both populations.

Most AFLP markers were mapped in the first or second cycle of map construction, thus showing reliable goodness of fit. These maps were retained for purposes of QTL mapping. However, a third cycle of map construction was used to force remaining makers into the map (Fig. 1). This high-density map provides a useful reference for fine genetic mapping efforts that are also under way. However, approximately 120 markers from both populations either would not fit or displayed exceedingly high  $\chi^2$  values in the third cycle of map construction. These markers were excluded from the published map (Fig. 1). In summary, the 164-sib TTC1 map

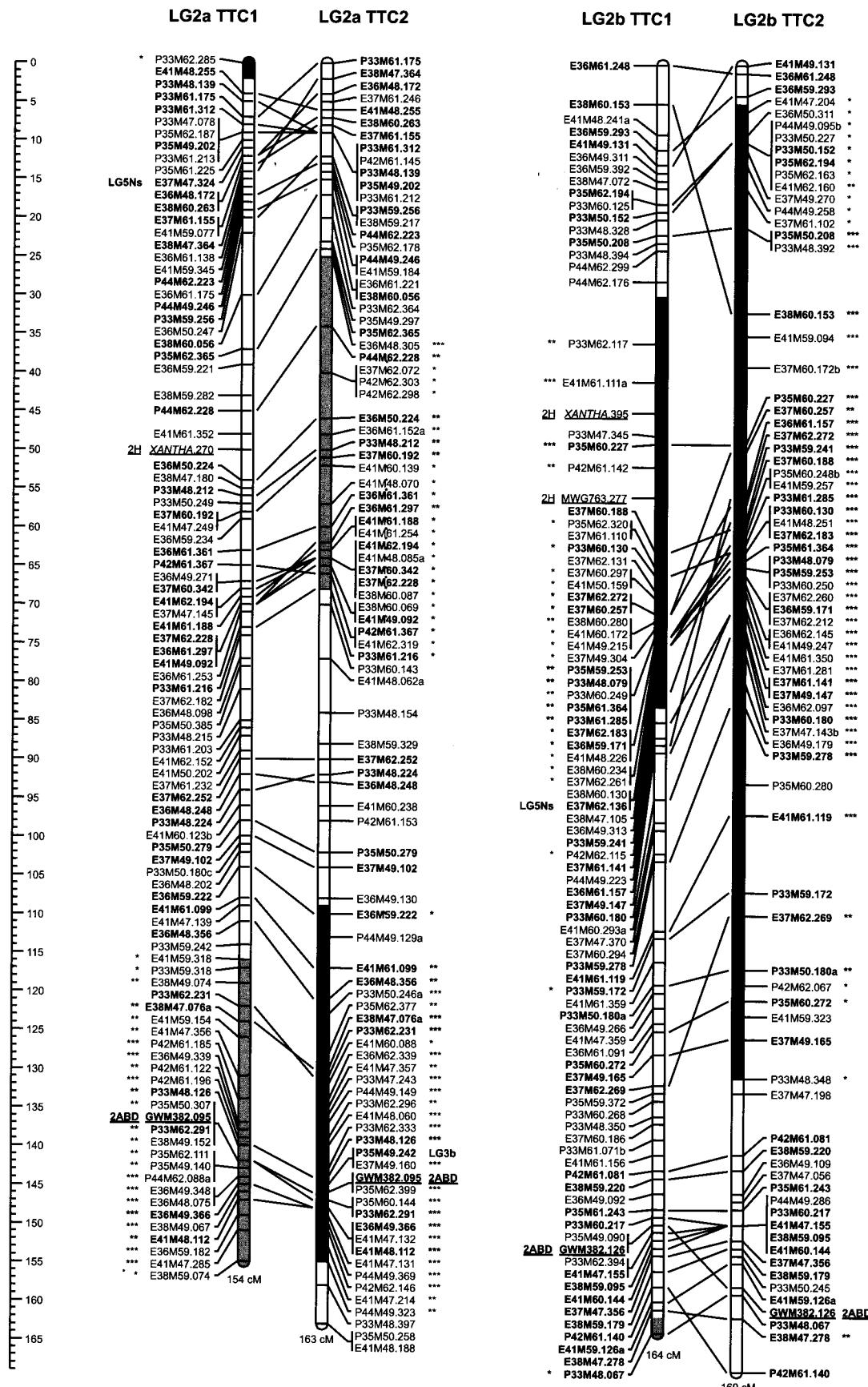
**Fig. 1.** Comparison of genetic linkage groups for two full-sib *Leymus* wildrye populations, TTC1 and TTC2. Molecular markers mapped in both TTC1 and TTC2 populations are indicated by bold text with line connections between homologous bridge loci or text reference to other non-homologous linkage groups. The SSR, STS, and RFLP anchor markers (underlined) are also identified by text reference to specific homoeologous chromosomes of wheat (ABD), barley (H), and rye (R) or corresponding oat linkage groups in parentheses (Table 2). Segregation distortion with an excess of *L. cinereus* alleles (dark shaded linkage blocks) or excess of *L. triticeoides* alleles (light shaded linkage blocks) are indicated by text reference (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; or \*\*\*,  $p < 0.001$ ). Genetic recombination distances (cM) are indicated on the left.



includes 1069 AFLP markers in 14 linkage groups spanning 2001 cM. The 170-sib TTC2 map contains 1002 AFLP markers in 14 linkage groups spanning 2066 cM. With 1583 AFLP markers mapped in TTC1 and (or) TTC2 (Table 1), the

average marker density is approximately 1 marker/1.2 cM with very few gaps greater than 10 cM in both populations (Fig. 1). Evaluation of map order ripples detected many competing, albeit subtle, alternatives. Nevertheless, the order

Fig 1. (continued).



**Fig 1.** (continued).

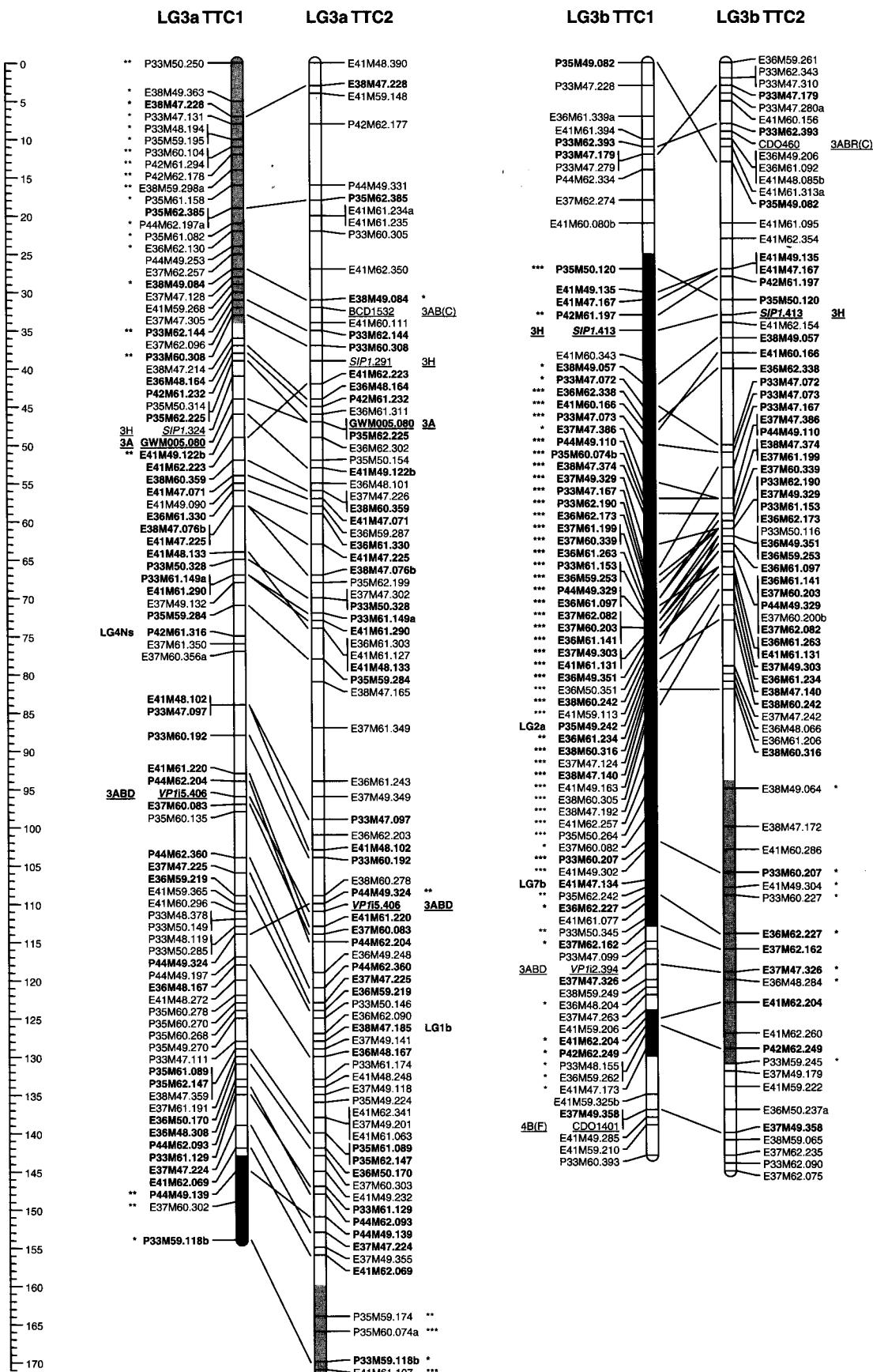
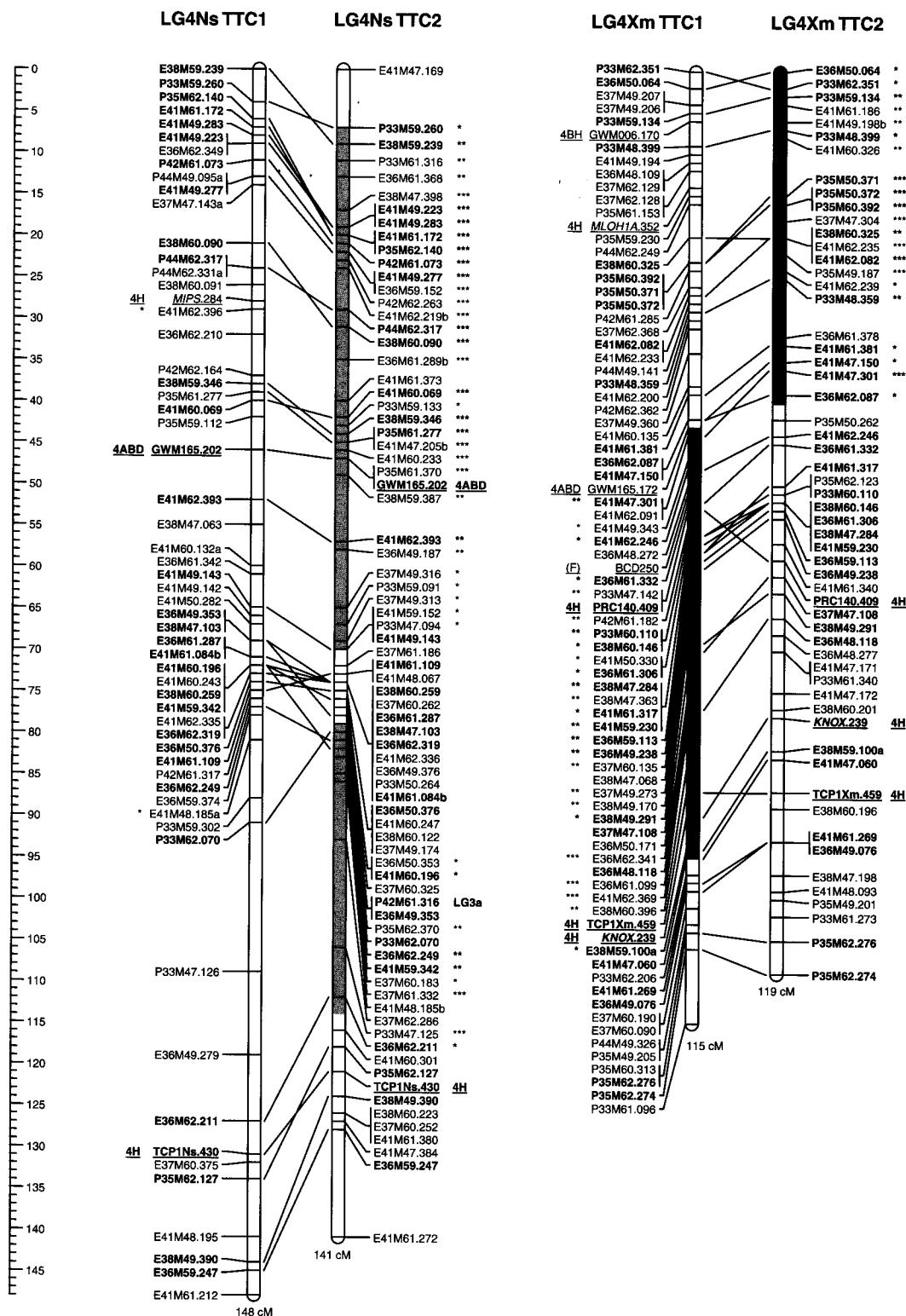


Fig 1. (continued).



of 488 AFLP homologous bridge markers and configuration of linkage groups is very similar in both populations (Fig. 1). Only 16 AFLP markers had the same apparent phenotype (capillary fraction) in TTC1 and TTC2, but map to different non-homologous chromosomes (Fig. 1). Given the similar pedigree of these two mapping populations, we do

not expect any substantial degree of chromosome rearrangement in these two populations. Thus, most differences in the genetic map order of molecular markers in TTC1 and TTC2 can be attributed to experimental uncertainty.

Markers that showed ambiguous affinities and (or) poor fit within linkage groups often displayed highly significant seg-

Fig 1. (continued).

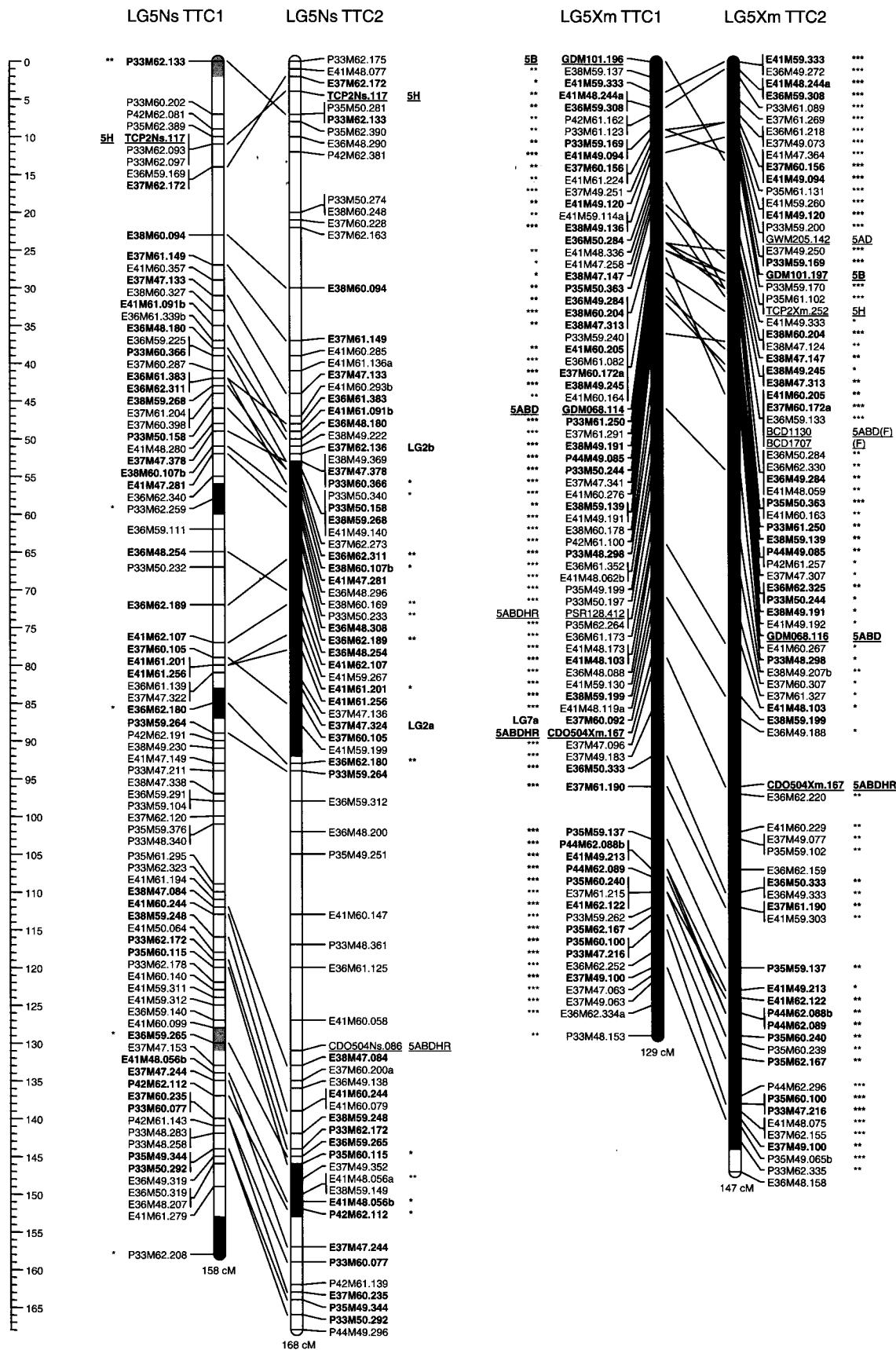
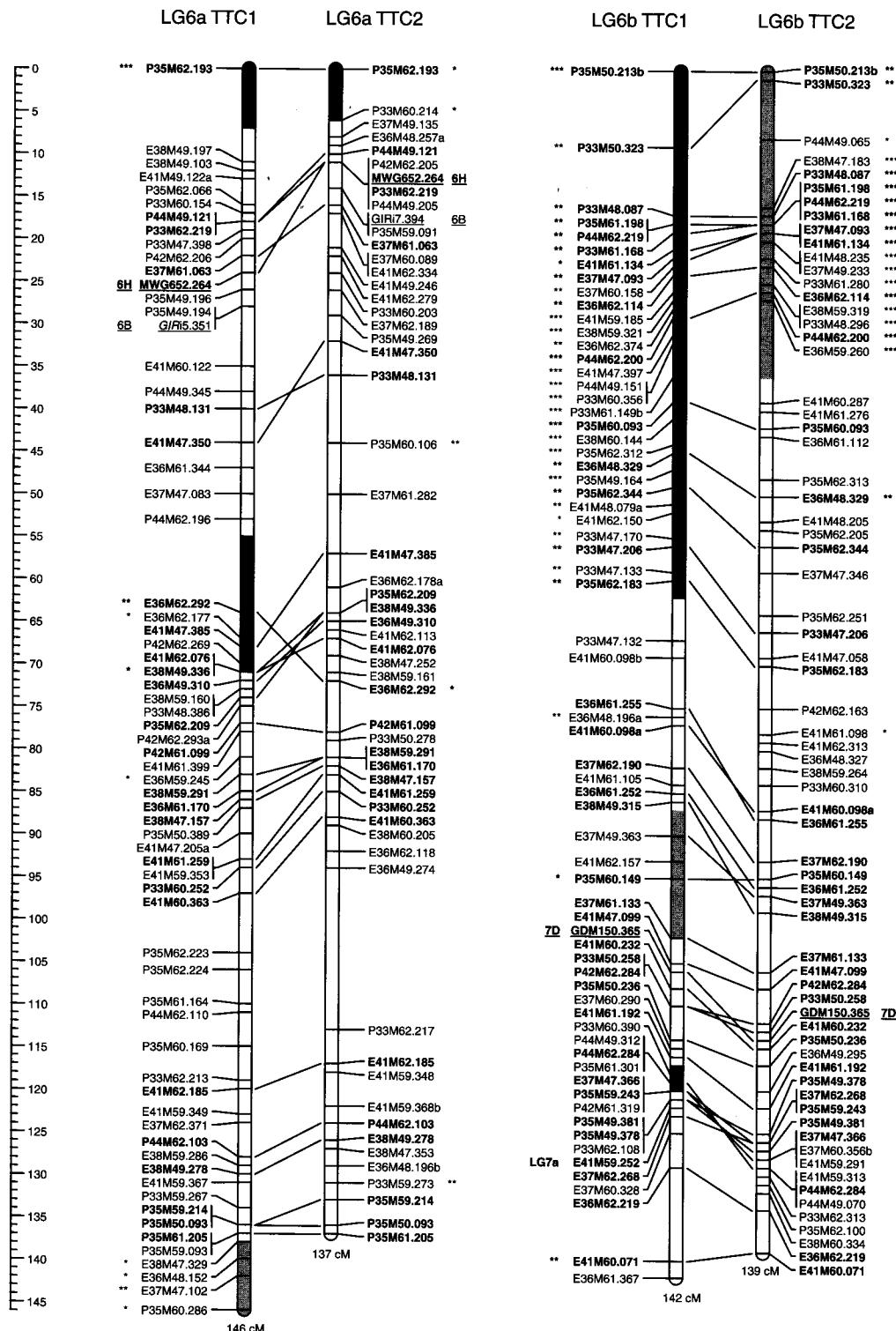


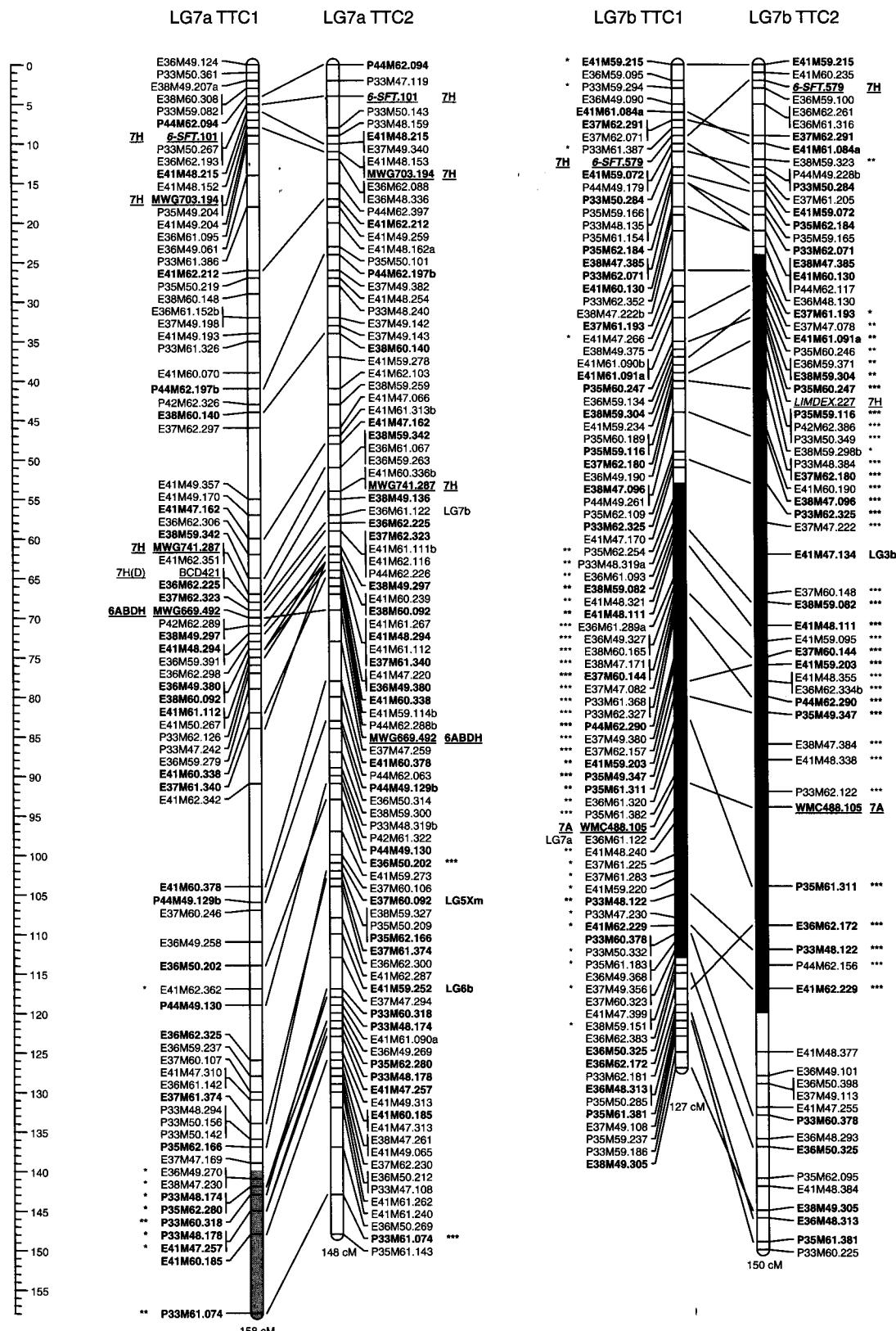
Fig 1. (continued).



regression distortion. A substantial number of these problematic data may have contained different fragment sequences (i.e., different loci) with the same apparent molecular size. However, segregation distortion was also observed for approximately 39 and 42% of the mapped loci in populations A and B, respectively (Fig. 1). These distorted loci represent at least 12 substantial linkage blocks in the TTC1

population and 11 substantial linkage blocks in the TTC2 population (Fig. 1). At least three of these chromosome regions showed the same pattern of segregation distortion in both TTC1 and TTC2 populations (Fig. 1). Skewed segregation ratios have been reported frequently in interspecific crosses for all types of markers: 69% in *Cryptomeria japonica* (Nikaido et al. 1999), 51 and 80% in *Lycopersicum* (Paterson

Fig 1. (concluded).



et al. 1991; De Vincente and Tanksley 1993), 36 (Xu et al. 1997) and 16% (Causse et al. 1994) in *Oryza*, and 30% in coffee (Ky et al. 2000). Thirteen unlinked chromosome re-

gions distributed over 7 of the 10 chromosomes showed significant deviations from Mendelian segregation in an interspecific *Sorghum* population (Chittenden et al. 1994). In

any case, segregation distortion caused by differential fitness combined with difficulties of data interpretation may contribute to ambiguous associations with different linkage groups. However, the average germplasm contribution of *L. cinereus* and *L. triticoides* was 52.7 (1022 cM) and 46.8% (908 cM), respectively, from the TC1 hybrid (0.5% missing data). Likewise, the average germplasm contribution of *L. cinereus* and *L. triticoides* was 53.2 (1062 cM) and 46.5% (928 cM), respectively, from the TC2 hybrid. Therefore, the TC1 and TC2 hybrids contributed approximately equal portions of *L. cinereus* and *L. triticoides* germplasm to the TTC1 and TTC2 mapping populations.

The linkage maps for TTC1 and TTC2 (Fig. 1) contain 6148 and 4925 singletons, respectively. Thus, 3.4 and 2.9% of the TTC1 and TTC2 mapping data were presumably genotyping errors. Excluding all singletons, approximately 12.9 and 14.9 recombination events were detected per TC1 and TC2 gamete, respectively. Dewey (1970) observed 14 bivalents, mostly rings, in 127 of 147 metaphase-I cells of a tetraploid *L. cinereus* × *L. triticoides* hybrid. Likewise, Hole et al. (1999) observed approximately 12 ring and 2 rod bivalents (26 chiasma) among allotetraploid hybrids of *L. flavescens* × *L. triticoides* and *L. flavescens* × *L. cinereus*. Therefore, approximately 13 recombination events per gamete would be expected based on these chiasma frequencies because only two of the four bivalent chromatids actually crossover at each chiasma. As expected (King et al. 2002), the average number of crossovers detected by AFLP linkage analysis of TC1 and TC2 pollen gametes corresponds 1:1 with the average number of recombinations expected based on chiasma frequencies observed in the pollen mother cells.

#### Anchored PCR markers and identification of homoeologous groups

The TC1, TC2, and T-tester parental genotypes were tested with 71 wheat or barley SSR primer pairs (excluding STS introns or exons that may also contain SSRs). Approximately 45 of these SSR primer pairs amplified products from *Leymus*, of which 11 primer pairs produced useful length polymorphisms that were mapped in TTC1 and (or) TTC2 (Table 2). Two SSR primer pairs, GWM382 and GWM165, detected more than one loci (Table 2; Fig. 1). Although the transferability rate of SSR markers among these divergent Triticeae species may be somewhat low (approximately 1/7 based on these results), there are many wheat and barley SSR sequences available. Moreover, testing SSR length variation is relatively easy compared with many other DNA genotyping procedures. Therefore, wheat and barley SSR primers may provide a useful source of anchor markers in other Triticeae species.

In addition to the 11 SSR markers, we also detected DNA length and (or) sequence polymorphism using 25 STS primer pairs (Table 2). Most of these STS markers were derived from well-characterized wheat and (or) barley loci, thus potentially useful anchor markers for *Leymus*. However, we also mapped *Leymus* and barley TCP sequences (Cubas et al. 1999) orthologous to the maize *teosinte branched 1* gene (Doebley 1997), and putative *Leymus* and barley serine (threonine) phosphatase (EC 3.1.3.16) sequences orthologous to the *Sorghum* PRC140 RFLP marker associated with rhizome proliferation of *S. propinquum* (Paterson

et al. 1995). Two orthologous STS loci were detected for the *XANTHA*, *SIP1*, *VPI*, TCP1, TCP2, CDO504, and 6-SFT gene sequences (Table 2; Fig. 1). Moreover, genome-specific primers were used to distinguish homoeologous TCP1, TCP2, and CDO504 loci (Table 2). Thus, several of these STS markers will be described in more detail.

The *Sorghum* PRC140 RFLP marker is located in the center of a major rhizome QTL region, on *Sorghum* linkage group C (Paterson et al. 1995), which is evidently orthologous to wheat homoeologous group 4 (Draye et al. 2001). A set of PCR primers designed from the *Sorghum* PRC140 rhizome cDNA sequence amplified a *Leymus* genomic DNA sequence that is homologous to the original PRC140 sequence and homologous to a putative *Sporobolus stapfianus* serine (threonine) phosphatase sequence (GenBank accession No. AJ242803). The initial *Leymus* PRC140 amplification products were monomorphic; however, primers designed from other regions of the putative *S. stapfianus* serine (threonine) phosphatase cDNA sequence amplified polymorphic genomic DNA sequences from TC1, TC2, and T-tester genotypes that were syntenous with five other homoeologous group 4 (LG4Xm) markers in the TTC1 and TTC2 mapping populations (Fig. 1). Moreover, several polymorphic *DpnII* restriction fragments from the corresponding barley STS amplification products were mapped to a locus fewer than 2 cM from ABC321 in the centromere region of barley chromosome 4H, using the *Hordeum vulgare* 'Steptoe' × *H. vulgare* 'Morex' mapping population (Kleinholz et al. 1993). The sequences and map positions of these barley and *Leymus* amplicons are evidently orthologous to the *Sorghum* PRC140 rhizome QTL marker (Draye et al. 2001).

The maize *tb1* gene controls the proliferation and elongation of lateral branches and tillers (Doebley 1997), which is developmentally similar to the proliferation and elongation of rhizomes in other grasses. The maize *tb1* gene encodes a putative transcription factor characterized by the basic Helix-Loop-Helix TCP and coiled-coil R domains, which probably mediate DNA binding and protein-protein interactions (Cubas et al. 1999). Two distinct *tb1*-like gene sequences, TCP1 and TCP2 (Fig. 2), were amplified from cDNA preparations from *L. triticoides* rhizomes using PCR primers designed in the conserved TCP and R domains of the maize *tb1* gene. Similarity between the *Leymus* TCP1 and maize *tb1* sequences is greater than similarity between *Leymus* TCP1 and TCP2 sequences (Fig. 2).

The barley TCP1 sequence was mapped near the HVM40, ABG003, and *Phy2* loci in the centromeric region of chromosome 4H by segregation analysis of the *H. vulgare* 'Harrington' × 'Morex' doubled-haploid mapping population developed by Hayes et al. (1997). Segregation of the 'Morex' TCP1 sequence (AF543434), amplified using the forward 5'-TCAATATGTCAAAGGGCG-3' and reverse 5'-GAGTTGGCAAACACCACTCC-3' primers, was detectable against the null 'Harrington' genotype. *Leymus* TCP1 sequences also map to homoeologous group 4 (Fig. 1). The *Leymus* TCP1Ns sequences correspond to the Ns genome of *Psathyrostachys juncea*, whereas the *Leymus* TCP1Xm sequences correspond to a genome that has not been conclusively identified (Fig. 3). Both LG4Ns and LG4Xm, distin-

**Table 2.** Description of SSR and STS anchor loci mapped in *Leymus*, with expected and observed amplicon sizes (bp).

Locus (group)	Primers	Gene and (or) repetitive sequence motif	Orthologous groups	Expected amplicon	<i>Leymus</i> amplicons	Mapped amplicons	References
GDM126 (LG1a) <i>HVA</i>	TCCATCATATCCGTAGGCCACA CGTGGTTGATTTCAGGAGT CATGGAGGGACAAACAC CGACCAAACAGGACTAAAGGA	Wheat Xgdm126 (CA) <sub>13</sub> Barley <i>HVA1</i> with (ACC) <sub>5</sub> X78205	1 D	181–189	146 <sup>a</sup> , 158 <sup>a</sup>	146 <sup>a</sup>	Pestova et al. (2000)
(LG1a) WMC336 (LG1b) <i>XANTHA</i>	(Svörlöf Weibull, unpublished)	Wheat WMC336 (GCC) <sub>7</sub> Barley protoporphyrin Mg-chelatase U26916	1 AD	112, 129	90 <sup>b</sup> , 101 <sup>a</sup> 160 <sup>a</sup> , 388 <sup>a</sup>	388 <sup>a</sup>	Straub et al. (1994) Ramsay et al. 2000 Wheat microsatellite consortium Jensen et al. (1996)
(LG2a, LG2b) GWM1382 (LG2a, LG2b) MWC763 (LG2b) <i>SIP1</i>	ATGGCAGAACCTAACGCCGA GTGTCCTCGGAAGACCCCTCG GTCAGATAAACGCCGCTCCAAT CTACGTGCACCAACCATTTG CATTCAATGTCAGGCACGG TAGCAGTCCAACAGCAGC CTTCGACACCATCACCCAG	Barley protoporphyrin Mg-chelatase U26916 Wheat Xgwm382 (GA) <sub>26</sub> Barley cMWG763 AJ234447 Barley <i>SIP1</i>	2 H	888	86–184 2 ABD	86–184 094 <sup>a</sup> ... 127 <sup>a</sup>	270 <sup>a</sup> <i>MboI</i> Röder et al. (1998)
(LG3a, LG3b) GWM005 (LG1a) <i>VPlz</i> (LG3b) <i>VPlz</i> (LG3a) <i>MPS</i>	ACCAAAATCGCATCGAACAT GCCAGCTACCTCGATAACACTC AGAAAGGGCCAGGGCTAGTAGT CAGAAGGTGCTCAAGCAGAG AGGTGAGTCTCGCTTCCCTT GGGTGATTTCATCGTGCTT GTGGGAGACTCTCTTGC GCCCTGTGTCATGGAGGGTGT CACACAGTTGCTCTTGAGA GACGACTTGTACAGGAGCA TGGAGATTAGAATCCACGCA GACGAGTTGATCACGAGGC TGGAGATTAGAATCCACGCA TGCAGTGGTCAGATGTTCC CTTTCTTCAAGATTGCC CGTATCACCTCTAGCTAAACT AGAGCCTTATCATGACCTACCTT ATGATACTCGTCTCCGTGCG GATATGAAGGCCACCCAGCAT GGGGAGGAGAAGGGTAGAGA ATGGCAGAAGGCTTGTCAATT AAGAAGAAAAGGCAAGCTCCC GATCTGCTCAGGTCAAGGC	(TCT) <sub>5</sub> M77475 Wheat Xgwm5, WMSS5 (TC) <sub>23</sub> (GT) <sub>12</sub> (GA) <sub>10</sub> Wheat <i>vp1</i> AJ400713 AJ400713 AJ400713 AJ400713 Barley <i>MPS</i> AF056325 <i>Leymus</i> TCP1 AF543437, AF543438 <i>Leymus</i> TCP1 AF543444, AF543441 Wheat Xgwm165 (GA) <sub>20</sub> Wheat Xgwm6 (WMS6) (GA) <sub>40</sub> Barley <i>Mlo-h1</i>	3 A	158–219	410 <sup>b</sup> 80 <sup>b</sup>	413 <sup>a</sup> 80 <sup>a</sup>	Röder et al. (1995)
(LG4Ns) TCP1Ns (LG4Ns) TCP1Xm (LG4Xm) GWM165 (LG4Ns, LG4Xm) GWM006 (LG4Xm) <i>MLOH1A</i> (LG4Xm) PRC140 (LG4Xm) <i>KNOX</i> (LG4Xm)	4 H	401	384 <sup>a</sup> ... 404 <sup>b</sup>	384 <sup>a</sup> , 394 <sup>a</sup>	McCarty et al. (1991)		
		3 ABD, 3 maize	70 <sup>b</sup>	406 <sup>a</sup> <i>HhaI</i>	Bailey et al. (1999)		
		3 ABD, 3 maize, 1 rice	694	284 <sup>a</sup> <i>HinfI</i>	McCarty et al. (1991)		
		4 H, 1 maize	452	50 <sup>b</sup>	Bailey et al. (1999)		
		maize, 3 rice	480–518	480–518 <sup>c</sup>	Larson et al. (1999)		
		4 H maize	451–463	451–463 <sup>c</sup>	Doebley et al. (1997)		
		4 H maize	188–191	172 <sup>a</sup>	Cubas et al. (1999)		
		4 ABD	202 <sup>a</sup>	172 <sup>a</sup> , 202 <sup>a</sup>	Doebley et al. (1997)		
		4 BH	196–207	170 <sup>a</sup>	Röder et al. (1998)		
		4 H	622	180 <sup>b</sup>	Ramsay et al. (2000)		
		4 H	60 <sup>b</sup>	352 <sup>a</sup>	Büsches et al. (1997)		
		4 H maize 1	662	1100 <sup>b</sup>	Neale et al. (2000)		
		4 H	228	239 <sup>a</sup> , 292 <sup>a</sup>	Dray et al. (2001)		
		4 H maize 1	564 <sup>a</sup> , 664 <sup>a</sup>	239 <sup>a</sup> , 564 <sup>a</sup>	Müller et al. (1995)		
		5 H	543	543 <sup>c</sup>	Vollbrecht et al. (1991)		
TCP2Ns (LG5Ns)	<i>Psathyrostachys</i> TCP-like gene AF543429	See Results	117 <sup>a</sup> <i>Tag1</i>	See Results			
	CTGGACCAAGACAATGA TGAACGGATGAAGTTGG						

Table 2 (*concluded*).

<sup>a</sup>Estimated by capillary electrophoresis (ABI3100) and Genescan software.

<sup>b</sup>Estimated by agarose gel electrophoresis.  
<sup>c</sup>Determined by DNA sequencing.

Determined by DNA sequencing

Barley limit dextrinase

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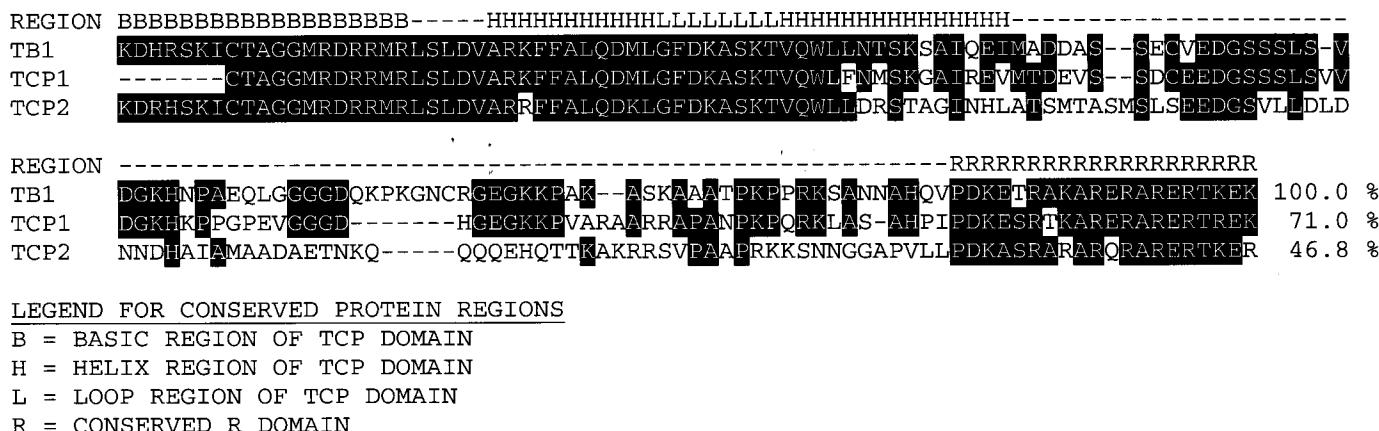
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**Fig. 2.** Alignment of conserved TCP and R domains of the maize *teosinte branched 1* (*tb1*) deduced amino acid sequence (GenBank accession No. U94494), the orthologous *Leymus* TCP1 deduced amino acid sequence (GenBank accession No. AF543437), and the homologous *Leymus* TCP2 deduced amino acid sequence (GenBank accession No. AF453427).



guished by the genome-specific TCP1Ns and TCP1Xm markers, contain a number of other anchor loci corresponding to Triticeae homoeologous group 4 including PRC140 (described above), *MIPS* (Larson and Ruboy 1999), and *KNOX* (Vollbrecht et al. 1991; Muller et al. 1995) (Fig. 1). Like *MIPS* and *KNOX*, the *tb1* gene maps to a region of maize chromosome 1 (Doebley 1997) that is evidently orthologous to Triticeae chromosome 4 (Ahn and Tanksley 1993; Nelson et al. 1995; van Deynze et al. 1995). Thus, sequence (Fig. 2) and mapping (Fig. 1) data indicate that the *Leymus* TCP1 genes are orthologous to the maize *tb1* gene.

The barley TCP2 sequences map 13 cM (LOD = 8.9) from MWG502 on the distal end of chromosome 5 HS by segregation analysis of the 'Harrington' × 'Morex' mapping population developed by Hayes et al. (1997). Segregation of the 'Harrington' (GenBank accession No. AF543430) and 'Morex' (GenBank accession No. AF543432) TCP2 sequences, amplified using the forward 5'-CTGAAACAA-GCAAGCAGCAG-3' and reverse 5'-GGATGGATGGATGGA-TGAAG-3' primers, was detectable by several *Hae*III restriction fragment polymorphisms. *Leymus* TCP2 sequences also mapped to homoeologous group 5 (Fig. 1). Thus, TCP2 sequences of barley and *Leymus* are homologous but not orthologous to the maize *tb1* gene. As might be expected, one of the *Leymus* TCP2 sequences (TCP2Ns) corresponds to the Ns genome of *P. juncea* (Fig. 3). The other *Leymus* TCP2 sequence (TCP2Xm) corresponds to a genome that has not been conclusively identified, although it is very similar to the *P. fragilis* TCP2 sequence (Fig. 3). Thus, similarity between the *P. fragilis* TCP2 sequence and *Leymus* TCP2Xm sequence was greater than similarity between the *P. fragilis* and *P. juncea* TCP2 sequences (Fig. 3). This observation suggests that *Leymus* may be a segmental allotetraploid, Ns<sub>1</sub>Ns<sub>1</sub>Ns<sub>2</sub>Ns<sub>2</sub>, as could be interpreted from other studies (Zhang and Dvorak 1991; Anamthawat-Jónsson and Bödvarsdóttir 2001). Unfortunately, we have not been able to amplify and compare other *P. fragilis* STS markers (Fig. 3). In any case, LG5Ns and LG5Xm were distinguished by genome-specific TCP2 and CDO504 STS markers (Table 2; Fig. 2). Satisfyingly, TCP2Ns and TCPXm

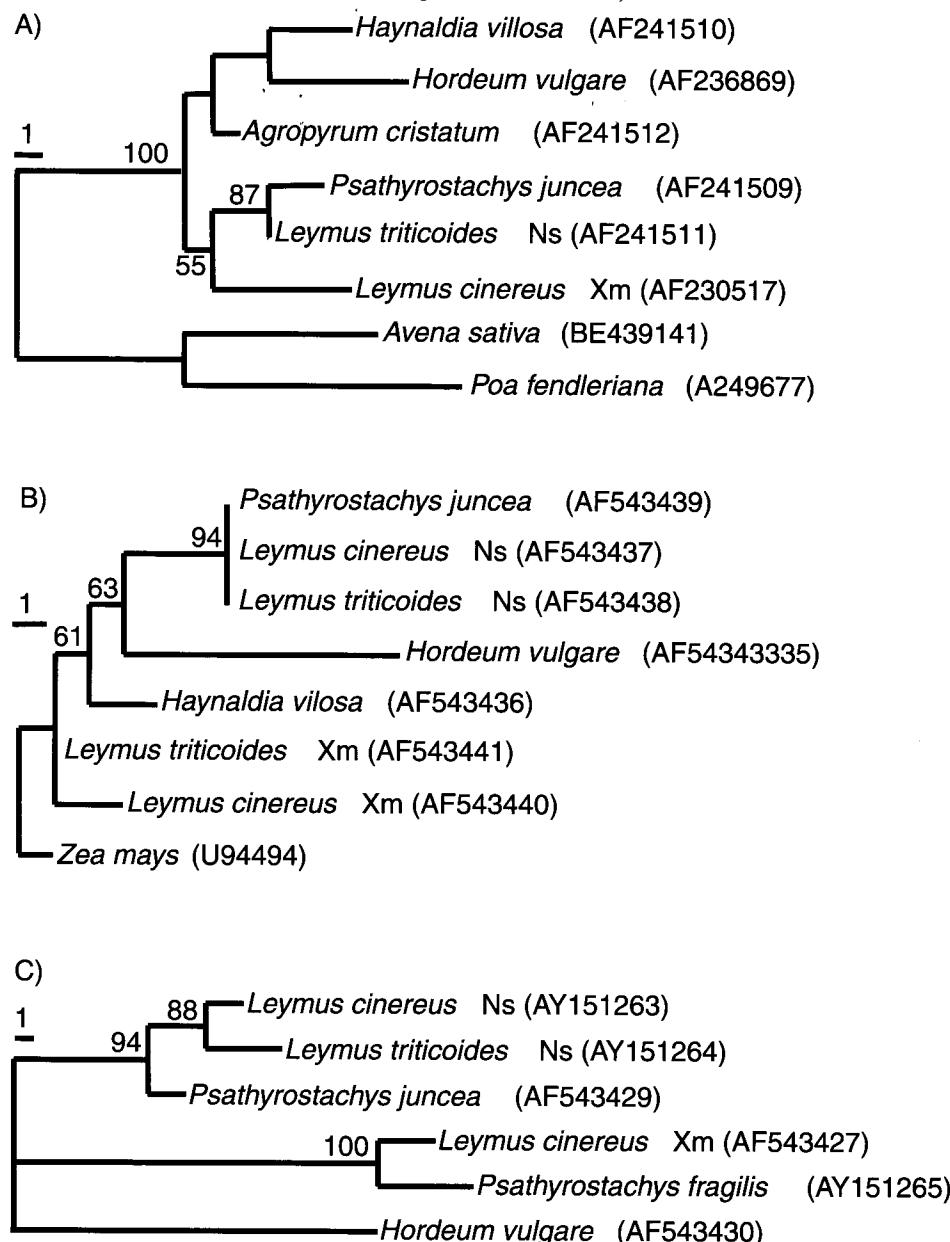
were coupled with genome-specific CDO504Ns and CDO504Xm markers, respectively (Fig. 2).

#### Identification of homoeologous groups

The 36 PCR primer pairs (Table 2) and 9 RFLP probes (Materials and Methods) detected 36 and 38 loci in the *Leymus* TTC1 and TTC2 mapping populations, respectively, with 24 bridge markers mapped in both populations (Fig. 1). Synteny of two or more anchor markers corresponding to each of the seven homoeologous Triticeae chromosomes was maintained among 12 of the 14 *Leymus* linkage groups (Fig. 1). However, only one putative anchor marker, WMC336, was detected for LG1b (Fig. 1). Likewise, only one putative anchor marker, GDM150, was detected for LG6b. Moreover, two linkage groups, LG3b and LG7a, included anchor markers corresponding to more than one homoeologous group. Specifically, LG7a contained four anchor markers from homoeologous group 7 and one anchored PCR marker, MWG669, from homoeologous group 6 (Fig. 1). Likewise, LG3b contained three anchor markers from homoeologous group 3 and one anchored RFLP marker, CDO1401, from homoeologous group 4 (Fig. 1). The *Leymus* MWG669 PCR marker was not sequenced, but the amplicon size was very close to the corresponding barley sequences. However, duplicated loci are commonly detected among the seven basic homoeologous groups of barley, wheat, and other Gramineae species. Thus, CDO1401 and MWG669 loci on *Leymus* LG3b and LG7a, respectively, may not be orthologous to the CDO1401 and MWG669 loci detected in wheat, barley, and oats.

Although *Leymus* may contain one or more chromosome translocations relative to other Triticeae species, the number of anchor markers here is insufficient to make any conclusions to either effect. Nevertheless, the synteny of putative anchored PCR and RFLP markers from the same homoeologous group is a useful starting point for the genetic mapping of functionally important genes in these perennial *Leymus* wildraces. Moreover, genome-specific TCP and CDO504 STS markers (Fig. 3) were used to distinguish homoeologous groups 4 and 5 (Fig. 1). At least one of the

**Fig. 3.** Phylogeny of sequence-tagged sites determined by parsimony analysis, with bootstrap confidence levels ( $n = 1000$ ). (A) A 244-bp overlapping region of oat CDO504 and orthologous Triticeae sequences located on homoeologous group 5 (trees = 1 of 7; length = 48; CI = 0.88). (B) A 212-bp overlapping region of the maize *tb1* gene and orthologous TCP1 Triticeae sequences located on homoeologous group 4 (tree = 1 of 1; length = 20; CI = 1.00). (C) A 351-bp overlapping region of orthologous TCP2 Triticeae sequences located on homoeologous group 5 (trees = 1 of 1; length = 64; CI = 0.97).



*Leymus* genomes is very similar to *Psathyrostachys juncea*. Although there is substantial evidence that *Leymus* may have other *Psathyrostachys* or *Psathyrostachys*-like ancestors, we chose to use Xm (unknown) as a conservative genome designation until this question can be more precisely and conclusively resolved.

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